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Nuclear DNA Normality of Spermatozoa Selected by Swim-up and Two-layer Percoll Gradient Techniques

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ABSTRACT

Objective To use acridine orange (AO) test to compare sperm DNA normality between washing and swim-up, and two-layer Percoll gradient techniques for both normal and abnormal semen samples.

Design Experimental study.

Setting Division of Infertility, Department of Obstetrics and Gynaecology, Faculty of Medicine, Siriraj Hospital, Mahidol University.

Subjects and methods Semen samples from 112 male partners of infertile couples were divided into two aliquots, which were separated by washing and swim-up, and two-layer Percoll gradient techniques. AO test was performed on the sperm before and after sperm separation.

Results The percentage of green-fluorescing sperm is improved after the two methods of sperm preparation but is more improved after two-layer Percoll gradient than washing and swim-up , both in normal and abnormal semen samples (89.0 ± 7.9 versus 81.9 ± 10.2, and 85.2 ± 10.2 versus 74.6 ± 14.8 ; $p < 0.001$ respectively).

Conclusion Two-layer Percoll gradient selected more sperm with nuclear DNA normality than washing and swim-up separation that may influence the outcome of assisted reproductive techniques.

Key words: nuclear DNA normality, acridine orange test, swim-up, percoll gradient

Routine semen analysis has long been the standard laboratory test of male fertility, but it cannot ascertain the functional capacity of the sample. Other practical tests for the clinical evaluation of male fertility are desirable. Recently, some comparatively simple sperm function tests, such as the hypoosmotic swelling test,[1] the triple - stain technique for evaluating the acrosomal reaction,[2] have been developed. Acridine orange (AO) staining is one of simple methods for assessing sperm nuclear normality. This dye produces green fluorescence when bound to double - stranded nucleic acids and red fluorescence with single - stranded nucleic acid.[3] Tejada et al[4] introduced a simplified method of AO

test, and suggested as a practical procedure to determine sperm quality during infertility investigations.

Various sperm separation techniques have been applied to select motile sperm fractions, free from seminal plasma, for assisted reproductive technologies.[5] The two widely used separation techniques in Thailand are washing and swim-up, and two-layer Percoll gradient. The latter technique has been demonstrated to be superior to the swim-up procedure for sperm recovery, enhanced motility, good fertilizing ability, improved sperm penetration assay, and better hypo-osmotic swelling test.[6-8] However, the effect of these two separation techniques on sperm nuclear DNA normality is rarely assessed.

The purpose of this study is to use AO test to compare sperm DNA normality between washing and swim-up, and two-layer Percoll gradient techniques for both normal and abnormal semen samples.

Materials and Methods

Semen samples

One hundred and twelve semen samples were obtained from male partners of infertile couples attending the Infertility Clinic, Siriraj Hospital, after 3-5 days of sexual abstinence. After liquefaction, they were examined according to the World Health Organization guidelines [9] and about 0.2 ml of each sample was smeared on precleaned slide and stained with acridine orange as described below. The remaining semen samples were divided into two aliquots, and sperm preparation was performed through swim-up and two-layer Percoll gradient procedures. After separation, the sperm derived from both techniques were smeared and stained for AO test.

Washing and swim-up

Two milliliters of human tubal fluid (HTF) culture medium supplemented with 10% human serum albumin (HSA) were added to 1 ml of the semen and mixed in a 15-ml sterile conical tube. Following centrifugation at 250xg for 10 minutes, the supernatant was decanted and the washing repeated. The pellet was gently layered over by 1 ml of the HTF medium and the sample was incubated at 37°C for 30-60 minutes in 5% CO_2. The upper part (0.5 ml) of the supernatant was aspirated for further analysis.

Two-layer Percoll gradient

The stock solution of Percoll was prepared by mixing 9 volumes of Percoll with 1 volume of 10 times concentrated Ham's F-10 medium. Further dilutions were made using HTF medium. A layer of 2 ml of 40% Percoll was layered over 2 ml of 80% Percoll in a 15-ml conical tube. One milliliter of the semen was gently layered over this gradient, and the tube was then centrifuged for 20 minutes at 600xg. The upper two layers were aspirated off until the 80% Percoll interface was reached. The remaining 80% layer at the bottom of the tube was resuspended in 2 ml of HTF medium and centrifuged for 10 minutes at 250xg. The final pellet was resuspended to a final volume of 0.5 ml of HTF.

Acridine orange staining [4]

Every sperm sample was smeared onto precleaned slide and allowed to air dry for 20 minutes. The smear then was fixed overnight in Carnoy's solution (methanol: acetic acid, 3:1), which was prepared daily. After fixation, the slide was allowed to dry for a few minutes before staining. The AO staining solution was prepared daily as follows: 10 ml of 1% AO in distilled water was added to a mixture composed of 40 ml of 0.1 M citric acid and 2.5 ml of 0.3 M $Na_2HPO_4.7H_2O$, pH 2.5. The sperm smear was stained for 5 minutes, and gently rinsed and mounted with distilled water. The slide was read the same day on a fluorescence microscope using a 490-nm excitation filter and a 530-nm barrier filter. A total of 300 cells were counted on each slide with the duration no longer than 40 seconds by the same investigator. Spermatozoa with a normal DNA content exhibited green fluorescence of the heads, while an abnormal DNA content was indicated by a spectrum varying from yellow-green to red.

Statistical analysis

Data were processed using the Statistical Analysis System. Wilcoxon's rank-sum test, the Kruskal-Wallis test and the chi-square test were used. The level of significance was set at $p < 0.05$.

Results

Semen characteristics of 112 samples are shown in Table 1. Based on WHO criteria,[9] there were 73 normal and 39 abnormal semen samples. The percentage of abnormality (34.8%) was corresponding to male factor of infertility in our clinic.

Table 2 shows that the percentage of green-fluorescing sperm after both techniques of sperm preparations was significantly higher than in original semen, both in normal and abnormal samples ($p<0.001$). Comparing between washing and swim-up, and two-layer Percoll gradient techniques, the latter separated significantly more green sperm than the former ($p<0.001$ in normal samples, and $p<0.01$ in abnormal samples). When comparing between normal and abnormal semen samples, the green sperm in normal group was significantly higher both in fresh semen and after swim-up ($p<0.01$ and <0.05 respectively). However, green sperm after two-layer Percoll gradient separation showed no significant difference between normal and abnormal groups ($p = 0.0648$).

Table 3 represents only 17 semen samples with all three parameters abnormality, oligoasthenoteratozoospermia, the green sperm also exhibited higher percentage after sperm preparations ($p < 0.001$). Two-layer Percoll gradient technique separated higher percentage of green sperm than washing and swim-up ($p < 0.05$).

Table 1. Comparison of semen characteristics between normal and abnormal samples *

Semen parameters	Normal samples (n = 73)	Abnormal samples (n = 39)
Volume (ml)	1.9 ± 0.6	2.1 ± 0.9
Sperm concentration ($x10^6$/ml)	68.5 ± 38.0	24.2 ± 16.8
Progressive motility (%)	60.5 ± 11.1	30.9 ± 12.3
Normal morphology (%)	37.8 ± 8.0	20.8 ± 8.5

* Values are means ± standard deviation

Table 2. Results of Acridine Orange (AO) testing before and after sperm preparation from normal and abnormal semen samples*

	Percentage of green-fluorescing sperm		
	Normal samples (n=73)	Abnormal samples (n=39)	P value**
Original semen	68.4 ± 14.3	58.7 ± 15.6	
	67(58.5 - 81.5)	56(48 - 72)	<0.01
Washing and swim-up	81.9 ± 10.2	74.6 ± 14.8	
	83(76.5 - 90.5)	78(67 - 85)	<0.05
2-layer Percoll gradient	89.0 ± 7.9	85.2 ± 10.2	
	91(83 - 95)	85(79 - 96)	NS
P value***	< 0.001	< 0.001	

* Values are means ± standard deviation and median (interquartile range ; IQR)
** Wilcoxon's rank - sum test
*** Kruskal - Wallis test

Table 3. Results of Acridine Orange (AO) testing from oligoasthenoteratozoospermic group (n = 17)

	Percentage of green-fluorescing sperm	
	mean ± SD	median (IQR)
Original semen	52.5 ± 12.7	51 (42 - 58.5)
Washing and swim-up*	66.8 ± 15.0	69 (53 - 81.5)
2-layer Percoll gradient*	78.8 ± 9.8	79 (70 - 85.5)

* $p < 0.05$

Discussion

During spermatogenesis the histones which bind to DNA in germinal cells become replaced gradually by intermediate proteins and by protamines that bind more tightly to DNA than do histones and result in compaction of chromatin in the nucleus of mature sperm.[10] AO testing was established to assess sperm nuclear DNA normality.[4] In the mature sperm nucleus, DNA associated with disulfide-rich protamines is resistant to denaturation by acid or heat and remains double stranded, and appears fluoresce green by AO staining.[11] Several authors suggested that a high ratio of green-fluorescing spermatozoa after AO staining indicated a high rate of 'fertile' cells, enabling the AO test to have potential application in routine infertility investigations.[4,11-13] In contrast to the study of Eggert-Kruse et al[14] which concluded that the AO testing cannot be recommended as a screening procedure for sperm quality and functional capacity. However, Hoshi et al[15] reported that semen samples in which 50% of spermatozoa exhibited green AO fluorescence consistently fertilized oocytes in their IVF patients, and none of the spermatozoa with < 50% green AO fluorescence produced a term pregnancy despite an average fertilization of 26% of oocytes.

In this study, the percentage of green-fluores cing sperm was generally similar to other reports.[4,16] The normal semen samples showed significantly higher green sperm than the abnormal group both in fresh semen and after swim-up separation. There was no significant difference of green AO - staining sperm between normal and abnormal groups after two-layer Percoll gradient separation. It demonstrated that even in abnormal semen samples, Percoll gradient technique could separated high percentage of sperm with normal DNA.

The present study showed that the percentage of green-fluorescing sperm was increased significantly with swim-up and Percoll gradient techniques but was higher after Percoll selection. This is consistent with the report of Colleu et al,[17] using electrophoretic analysis of nucleoproteins, showed that nuclear maturity of spermatozoa was improved after swim-up and Percoll gradient selection but was more improved after Percoll. Golan et al,[18] using acridine orange staining and flow cytometry, also showed that the chromatin quality of spermatozoa was improved by swim-up and Percoll gradient centrifugation methods. Our study demonstrated that even in the oligoasthenoteratozoospermic samples, the nuclear DNA normality was significantly more improved after two-layer Percoll gradient than after washing and swim-up techniques.

In conclusion, two-layer Percoll gradient selected more sperm with nuclear DNA normality than washing and swim-up separation that may influence the outcome of assisted reproductive techniques.

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